Maternal Gametophyte Effects on Seed Development in Maize

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ABSTRACT Flowering plants, like placental mammals, have an extensive maternal contribution toward progeny development. Plants are distinguished from animals by a genetically active haploid phase of growth and development between meiosis and fertilization, called the gametophyte. Flowering plants are further distinguished by the process of double fertilization that produces sister progeny, the endosperm and the embryo, of the seed. Because of this, there is substantial gene expression in the female gametophyte that contributes to the regulation of growth and development of the seed. A primary function of the endosperm is to provide growth support to its sister embryo. Several mutations in *Zea mays* subsp. *mays* have been identified that affect the contribution of the mother gametophyte to the seed. The majority affect both the endosperm and the embryo, although some embryo-specific effects have been observed. Many alter the pattern of expression of a marker for the basal endosperm transfer layer, a tissue that transports nutrients from the mother plant to the developing seed. Many of them cause abnormal development of the female gametophyte prior to fertilization, revealing potential cellular mechanisms of maternal control of seed development. These effects include reduced central cell size, abnormal architecture of the central cell, abnormal numbers and morphology of the antipodal cells, and abnormal egg cell morphology. These mutants provide insight into the logic of seed development, including necessary features of the gametes and supporting cells prior to fertilization, and set up future studies on the mechanisms regulating maternal contributions to the seed.

KEYWORDS maize; embryo sac; maternal effect; endosperm transfer layer; gametophyte

THE process of double fertilization is unique to flowering plants and results in the formation of the seed. The two sperm cells of the pollen grain fertilize the egg and central cell of the female gametophyte, or embryo sac, to form the diploid (1 maternal:1 paternal) embryo and typically triploid (2 maternal:1 paternal) endosperm, respectively (Sheridan and Clark 1994; Walbot and Evans 2003). The endosperm is thus a genetic sister of the embryo and is functionally equivalent to the mammalian placenta, acting as a nutritive tissue that supports the growth of the developing embryo and seedling. The maize endosperm consists of several morphologically and transcriptionally distinct domains: the aleurone, the basal endosperm transfer layer (BETL), the starchy endosperm, the conducting zone (CZ), the basal intermediate zone (BIZ), and the embryo-surrounding region (Olsen *et al.* 1999; Olsen 2004; Leroux *et al.* 2014; Li *et al.* 2014).

Haploid gene expression and patterning of the female gametophyte prior to fertilization can significantly affect the development of both the endosperm and embryo (Drews *et al.* 1998; Walbot and Evans 2003; Marton *et al.* 2005; Vernoud *et al.* 2005). The maize embryo sac is produced from a single megaspore by three rounds of free nuclear divisions generating an eight-nucleate syncytium which then cellularizes to produce seven cells of four types (Evans and Grossniklaus 2009): the egg cell, two synergids, the central cell, and three antipodal cells. Division of the antipodal cells, associated with auxin signaling, produces a cluster of 20–100 antipodal cells in maize (Chettoor and Evans 2015). The function of the

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antipodal cells is undetermined but they are hypothesized to act as a transfer tissue based on the presence of cell wall invaginations on the surfaces facing the maternal nucellus (Diboll 1968). Alternatively, they could act as a signaling center by providing positional information for the embryo sac, or even for the endosperm since they persist in the maize seed after fertilization (Weatherwax 1926; Randolph 1936).

Two types of mutants with maternal effects on seed development can be distinguished based on their mode of inheritance: those in genes required in the maternal sporophyte (Li and Berger 2012; Li and Li 2015) and those in genes required in the maternal female gametophyte (Luo *et al.* 2014). They can be distinguished from each other by the mode of transmission (Grossniklaus and Schneitz 1998; Evans and Kermicle 2001). Recessive maternal sporophyte effect mutants will only have consequences if parent plants are homozygous. Both maternal gametophyte effect mutants and dominant maternal sporophyte effect mutants produce abnormal seeds when heterozygotes are crossed as females; but in the case of gametophyte mutants the abnormal seeds inherit the mutant allele because the embryo sac must carry the mutation to cause an effect, while the allele present in the embryo sac (and hence the seed) is irrelevant in the case of maternal sporophyte effects. Consequently, in the case of dominant maternal sporophyte effects, wild-type and mutant alleles are equally represented in both abnormal and normal seeds.

Gametophytic maternal-effect mutants have been identified in both *Arabidopsis* and maize (Gavazzi *et al.* 1997; Evans and Kermicle 2001; Grini *et al.* 2002; Olsen 2004; Köhler and Grossniklaus 2005; Pagnussat *et al.* 2005; Gutierrez-Marcos *et al.* 2006b; Pien and Grossniklaus 2007; Phillips and Evans 2011). Although not affecting postfertilization seed development when transmitted through the pollen, many gametophytic maternal-effect mutations in *Arabidopsis* (Pagnussat *et al.* 2005; Boavida *et al.* 2009) and maize (Evans and Kermicle 2001; Gutierrez-Marcos *et al.* 2006b; Phillips and Evans 2011) have reduced male transmission, indicating a separate role for the gene in pollen development/function.

Studies of these mutants have revealed several causes for maternal effects, as identified through genetic and cellular analysis. Maternal gametophyte effects can be caused by defects in functional gene dosage in the endosperm (Singletary *et al.* 1997), embryo-sac morphology (Lin 1978), cytoplasmic storage of gene products (Springer *et al.* 2000), and imprinting (Kinoshita *et al.* 1999; Vielle-Calzada *et al.* 1999). Sporophytic maternal effects can occur through disruption of maternal transfer tissues or integuments (Felker *et al.* 1985; Garcia *et al.* 2005), nonreduction of gametes (which leads to endosperm parental ploidy imbalance) (Barrell and Grossniklaus 2005; Singh *et al.* 2011), or microRNA production (Golden *et al.* 2002). Although the two types of maternal effects have distinct modes of inheritance and time of action, there is evidence of interaction between the imprinting pathway (typically a gametophyte effect) and maternal sporophyte effects (Dilkes *et al.* 2008; FitzGerald *et al.* 2008).

Nonequivalence of the maternal and paternal genomes in endosperm development was identified through the analysis of interploidy crosses (*e.g.*, tetraploid by diploid) in multiple species of plants, and these data contributed to the formation of the parental conflict theory (Haig and Westoby 1989). According to this theory, maternal and paternal alleles in the endosperm have different activities, leading to restriction or promotion of the growth of the endosperm, respectively. The endosperm phenotypes of seeds with maternal or paternal genome excess are in agreement with this theory (Haig and Westoby 1991; Charlton *et al.* 1995; Scott *et al.* 1998). In maize, the BETL is particularly sensitive to maternal or paternal genome excess (Charlton *et al.* 1995). Nonequivalent expression of the parental alleles of many genes is present in the embryo as well, primarily before the midglobular stage (Vielle-Calzada *et al.* 2000; Baroux *et al.* 2001; Grimanelli *et al.* 2005; Autran *et al.* 2011; Baroux and Grossniklaus 2015). Analysis of the early phenotypes of embryo-lethal mutants corroborated these studies and demonstrated that early embryogenesis is largely under maternal control (Del Toro-De León *et al.* 2014).

RNA sequencing has enabled the identification of hundreds of genes with parent-specific and parent-biased expression in the seed of several plant species (Gehring *et al.* 2011; Hsieh *et al.* 2011; Waters *et al.* 2011; Wolff *et al.* 2011; Zhang *et al.* 2011; Xin *et al.* 2013; Pignatta *et al.* 2014). Many genes have only a subset of their naturally occurring alleles imprinted. Frequently, imprinting is stage-specific, with expression being uniparental early in endosperm development and biallelic later. Gametophytic maternal-effect mutants in *Arabidopsis* frequently show defects during this early period of development (Pagnussat *et al.* 2005; Ngo *et al.* 2012).

The imprinted status of these genes is regulated, at least in part, by parent-specific DNA methylation, polycomb group-mediated repression, and small RNA pathways (Köhler *et al.* 2003; Gutierrez-Marcos *et al.* 2004; Köhler *et al.* 2005; Gutierrez-Marcos *et al.* 2006a; Haun and Springer 2008; Makarevich *et al.* 2008; FitzGerald *et al.* 2009; Jahnke and Scholten 2009; Hsieh *et al.* 2011; Wolff *et al.* 2011; Vu *et al.* 2013; Pignatta *et al.* 2014; Zhang *et al.* 2014); and is often associated with repetitive DNA elements (Gehring *et al.* 2009; Villar *et al.* 2009; Pignatta *et al.* 2014). Molecular mechanisms that mark and maintain silenced alleles include a complex interplay between DNA methylation and histone modifications (Kawashima and Berger 2014).

While no functional data are available for most imprinted genes in plants, the maize maternally expressed *meg1* gene has been shown to promote nutrient allocation to the seed by promoting differentiation of the BETL (Costa *et al.* 2012). However, the promotion of endosperm growth by a maternally active gene is the opposite of that predicted by parental conflict theory and demonstrates that there is maternal control of essential seed developmental processes unrelated to parental conflict theory. A different explanation for the function of imprinting in the seed is to generate functional diversity of genes in seed development (Bai and Settles 2014;

Pignatta *et al.* 2014). As these models are not mutually exclusive, selective pressure from both mechanisms (and others) could be operating during evolution to generate parent-of-origin-specific expression of genes for different purposes in the seed. For example, some paternally expressed genes are important for establishing interploidy crossing barriers (Kradolfer *et al.* 2013; Wolff *et al.* 2015), while others are important for patterning of the embryo (Bayer *et al.* 2009; Costa *et al.* 2014).

Most maternal-effect mutants described in *Arabidopsis* do not have any prefertilization morphological defects (Grini *et al.* 2002; Pagnussat *et al.* 2005), except for those with fertilization-independent seed development (Ohad *et al.* 1996; Chaudhury *et al.* 1997; Grossniklaus *et al.* 1998; Kiyosue *et al.* 1999). Some of the maternal-effect mutants in maize have abnormal gametophyte morphology that may contribute to their effects on seed development and pollen transmission (Gutierrez-Marcos *et al.* 2006b; Phillips and Evans 2011). Here we describe a set of maternal-effect mutants in maize with varying effects on seed development. A majority have visible morphological defects in the embryo sac before fertilization, and an overlapping majority affect patterning of BETL gene expression in the endosperm after fertilization. In most cases, the prefertilization defects are sufficient to explain the defects in seed development. Consequently, only a subset of these mutations may affect imprinted genes or imprinting processes. Whether or not any of these mutations have imprinting-specific effects or affect both imprinting and prefertilization embryo-sac development will be resolved after cloning and molecular analysis of the affected genes.

Materials and Methods

Plant material and growth conditions

This collection of maize *maternal-effect mutants* (*mem*) was developed from a variety of mutagenesis populations as follows: The *Mn-Uq* mutant was isolated previously (Pan and Peterson 1989). The *sans scion1* (*ssc1*), *heirless1* (*hrl1*), *no legacy1* (*nol1*), *baseless2* (*bsl2*), and *topknot1* (*tpn1*) mutants were identified as rare ears with 25–50% defective kernels after pollination of females with wild-type males during routine propagation of maize genetic stocks. The *ssc1* mutation arose in a W22 inbred maize (*Zea mays*) plant carrying a mutable allele of *enhancement of r1* (*enr1*) and a pale-aleurone-conferring allele, *R1-r*::*(Venezuela)*, of the *r1* gene (Stinard *et al.* 2009). *hrl1* arose in a W64A inbred line with active *Mutator* (*Mu*) transposons. *nol1* arose in a line with active *Ac/Ds* transposons from a seed carrying a revertant to wild type of a *vp1-m1*::*Ds* mutant allele. *bsl2* arose in an active *Mu* W64A/A158 hybrid line. *topknot1* (*tpn1*) arose in an active *Mu* B73/W23 hybrid line. Two mutants, *superbase1* (*sba1*) and *maternally reduced endosperm1* (*mrn1*), were identified from an EMS mutagenesis as rare ears with a high frequency of defective kernels in an open pollinated population. One mutant, *hrl2*, arose as a single defective kernel event in a W22 inbred line with active *Ac/Ds* transposons. The other mutants arose in *UniformMu* maize lines, inbred W22 (McCarty *et al.* 2005), as single defective kernel events on otherwise wild-type ears. Mutants were typically propagated as heterozygotes by transmission through the female and selection for miniature or defective kernels. Mutants and wild-type controls were grown side by side for each experiment, either in summer field conditions or in greenhouses under long-day conditions (16 hr light:8 hr dark cycles).

Most mapping populations were generated by crossing *mem*/+^{Mo17} or *mem*/+^{B73} hybrid females to wild-type Mo17 or B73 males, respectively. For *hrl1* and *bsl2*, the mutant phenotype was suppressed in F_1 hybrids with B73 and Mo17, so the mapping populations were generated by crossing *mem*/+^{B104} females to wild-type B104 males. For *hrl2*, the mapping population was generated by crossing *hrl2*^{W22}/+^{W64A} females to wild-type W64A males.

Molecular mapping

DNA was extracted from seedlings by minor modification of the method of Saghai-Maroof *et al.* (1984) or from mature seeds (Martin *et al.* 2010), and PCR reactions were performed as described (Evans and Kermicle 2001). Initial map position was determined from bulk segregant analysis by comparing DNA from a pool of 48 normal seeds (mostly wild-type homozygotes) to DNA from a pool of 48 defective seeds (mutant heterozygotes), using either SNP-based Sequenom mapping (Liu *et al.* 2010) or PCR with a set of polymorphic SSR markers (Martin *et al.* 2010). When bulk segregant analysis showed heterozygosity in the mutant pool but near homozygosity in the wild-type pool, PCR was performed using the same SSR markers or nearby SSR markers on 48 defective and 48 normal kernel individuals to verify cosegregation with the mutant phenotype. Map position was refined using additional SSR and indel PCR-based markers within the appropriate chromosomal interval.

Transmission and viability assays

For *ssc1*, map position was first identified based on linkage to the visible kernel mutant *yellow endosperm1* (*y1*). Male and female transmission of the *ssc1* mutation and penetrance of the defective kernel phenotype were partially assessed using plants carrying *ssc1* linked in repulsion phase to *y1*. The genetic distance between *ssc1* and *y1* was determined using the kernel phenotypes of *y1* and *ssc1*. Transmission of *y1* was observed after making reciprocal crosses between + *y1*/*ssc1* + plants and homozygous *y1* plants. Similarly, for *stt3* and *mrn2*, map position was identified based on linkage to the *r1* gene. Male and female transmission of *stt3* and *mrn2* and penetrance of the defective kernel phenotype were partially assessed using reciprocal crosses between heterozygous *mem R1-r*::*standard*/+ *r1-r* plants and homozygous *r1-r* plants. For all other mutants, normal kernels from reciprocal crosses between mutant heterozygotes and wild-type plants were grown to maturity and progeny tested to determine what fraction had inherited the wild-type allele (*i.e.*, were homozygous wild type), and what fraction had inherited the mutant allele (*i.e.*, were heterozygous). For male crosses, this frequency produces the male transmission rate. For the female crosses, this frequency is

combined with the frequency of the defective kernels to calculate the female transmission rate using the percentages of all kernels that are homozygous wild type, all that are defective, and all that are heterozygous mutant but appear normal. To calculate the percentage of embryo sacs carrying the mutation that produced a detectable kernel (whether defective or normal), it was assumed that half of the embryo sacs inherited the mutation. If fewer than half of all kernels were mutant heterozygotes, then the number of embryo sacs that would need to be added to make the number of homozygous wild-type and heterozygous mutant kernels equal was assumed to consist of mutant embryo sacs that did not produce a detectable kernel. For viability assays, defective kernels, regardless of severity, were germinated on filter paper. If necessary, growing shoot tips were liberated by making an incision in the pericarp just beyond the tip of the shoot. Seeds with any root or shoot growth were transplanted to soil in small pots, and survivors were transplanted at the two- to three-leaf stage to the field and grown to maturity.

Confocal microscopy and histology

Embryo sacs were analyzed from mutant heterozygotes at mature stage (with a silk length ≥20 cm). Samples were processed and visualized on a Leica SP5 or Leica SP8 (Wetzlar, Germany) laser scanning confocal microscope as described previously (Gutierrez-Marcos *et al.* 2006b; Phillips and Evans 2011). Excitation was performed at 405, 488, and 561 nm and emission was collected at 410–480, 495–555, and 565–730 nm for the merged images. Images were analyzed and processed using ImageJ (National Institutes of Health) and Adobe Photoshop CS3. Figures were produced by generating a single image from a projection of all optical sections containing embryo-sac nuclei.

For the reporter assays, mutant heterozygotes were crossed as females by males either hemizygous or homozygous for $_{Pro}$*Bet1*::β-*glucuronidase* (*GUS*) (Hueros *et al.* 1999). For some mutants, additional GUS assays were performed on kernels from crosses of females heterozygous for the mutation and hemizygous for the $_{Pro}$*Bet1*::*GUS* transgene after pollination by wild type. If possible, the mutant line was crossed with $_{Pro}$*Bet1*::*GUS* lines in the same maize-inbred background as the mutant. In some cases, fewer than half of the normal progeny of the $_{Pro}$*Bet1*::*GUS* hemizygotes expressed the transgene as if it were silencing. Consequently, mutant kernels were always compared to their normal siblings. Normal and mutant kernels were bisected along the longitudinal axis, cutting the embryo in half, and stained overnight for GUS activity as previously described (Gutierrez-Marcos *et al.* 2006b). In kernels with indistinct embryos, the silk attachment point was used to determine the midline of the germinal face of the kernel. Images were collected on a Nikon (Garden City, NY) Eclipse E600 UV fluorescent microscope equipped with a Fujifilm FinePix S5 Pro camera.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. Seeds of mutant lines are available upon request.

Results

Isolation of maternal-effect mutants

A total of 16 mutants with maternal gametophyte effects on seed development were collected and verified using reciprocal crosses and progeny testing to verify their strict maternal effects on seed development, and that these maternal effects depended on the genotype of the embryo sac (Figure 1). Five mutants arose spontaneously in standard maize stocks rather than as part of a screen for maternal-effect mutants. The *ssc1*, *hrl1*, *bsl2*, *nol1*, and *tpn1* were first discovered as heterozygous plants segregating defective kernels, despite females being crossed with a wild-type male. Consequently, they were not expected to be recessive, zygotic mutants, but had to be maternal-effect mutants or dominant-zygotic mutants. *ssc1* arose in a standard W22 inbred line carrying a mutable allele of *enr1* (Stinard *et al.* 2009). In combination with certain alleles of *r1*, the *enr1* mutation leads to more intense pigmentation in the aleurone of the germless mutant kernels than their normal siblings (Figure 1B). This facilitated identification of the *ssc1* mutant plant, which would not otherwise have been as obvious because the embryos are covered and the endosperms are normal in appearance. The *hrl1*, *bsl2*, and *tpn1* mutants all arose in lines with active *Mutator* transposons, and *nol1* arose in a line with active *Ac/Ds* transposons. The *sba1* and *mrn1* mutants were identified as heterozygous plants producing defective kernels when pollinated by wild-type males from a population of EMS-mutagenized families. *ssc1* mutant kernels were not viable but the mutant was recovered from the normal siblings, a fraction of which carried the *ssc1* mutation. The other mutants were recovered by growing the few viable defective kernels to maturity and making reciprocal crosses with them.

To screen for new, viable maternal-effect mutants, we took advantage of the fact that *Mu* and *Ds* insertions occur late in flower development and often affect only a single ovule. Each seed from a *Mu* active or *Ac/Ds* female can then be treated as a potentially independent event. Rare defective seeds were collected from *Mu* active *UniformMu* W22 females and *Ac/Ds* W22 females that otherwise produced normal seed (Supplemental Material, Figure S1). These could arise because of environmental reasons, because of aneuploidy in the seed, because of a new dominant zygotic mutation in the seed, or because of a new maternal-effect mutation in the embryo sac giving rise to the seed. Two dominant zygotic kernel mutants were isolated as part of these screens.

A total of 566 defective kernels of various types were collected from ~750 *UniformMu* ears. These ears were generated by crossing *Mu* active *UniformMu* females (*MuDR*; *bz1-mum9*::*Mu*; *R1*; W22) with males of lines without *Mu* activity (*Mu* off) (*bz1-mum9*::*Mu*; *R1*; W22 without *MuDR*). Defective seeds were germinated on filter paper and planted to soil after

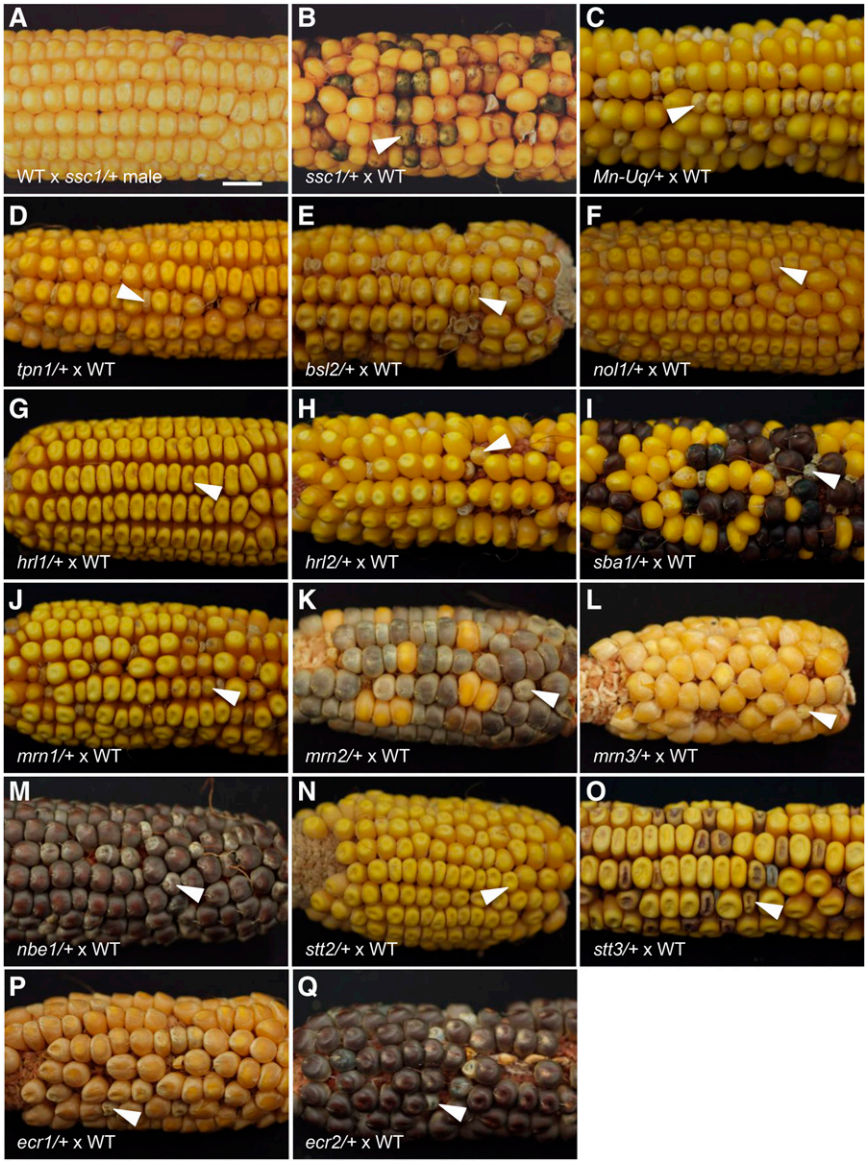

Figure 1 Ears of maternal-effect mutants. (A) Wild-type W22 female pollinated by *+/ssc1*; *enr1-m* with all normal kernels, showing the strict maternal effect. (B–Q) Maternal-effect mutant heterozygous females crossed by wild-type pollen. (B) *+/ssc1*; *enr1-m* W22 female by wild-type male. Germless mutant kernels with intense pigmentation of *R1-r*::*(Venezuela)*. (C) *+/Mn-Uq* W22. (D) *+/tpn1* Mo17. (E) *+/bsl2* B104. (F) *+/nol1* B104. (G) *+/hrl1* B104. (H) *+/hrl2* W22. (I) *+/sba1* W22. (J) *+/mrn1* B73. (K) *+/mrn2* W22. (L) *+/mrn3* W22. (M) *+/nbe1* W22. (N) *+/stt2* B73. (O) *+/stt3* Mo17, note the linkage between the small kernel phenotype and *R1-r*::*standard* (purple/brown kernels). (P) *+/ecr1* W22. (Q) *+/ecr2* W22. Arrowheads indicate representative defective kernels on each ear. Ear tips and hence the germinal sides of the kernels are all to the left. Bar, 1.0 cm. WT, wild type.

root and shoot emergence. A total of 124 of the defective seeds produced viable seedlings and ~20% of the survivors produced morphologically abnormal, male sterile plants consistent with aneuploid or haploid syndromes. Reciprocal crosses were made between the remaining 97 plants and a *Mu* off line to determine if the defective kernel phenotype was heritable and whether it behaved as a dominant zygotic mutant [*i.e.*, defective kernels were produced when crossed both as males or females (Figure S2)] or as a maternal-effect mutant (*i.e.*, defective kernels were produced when crossed as a female but only normal kernels were produced when crossed as a male). Eight plants that produced >5% defective kernels as females were retested in the next generation. Seven heritable maternal-effect mutants were identified from these. These mutants include *no bet1 expression1* (*nbe1*), *stunter2* (*stt2*), *stt3*, *mrn2*, *mrn3*, *empty creche1* (*ecr1*), and *ecr2*.

A similar mutagenesis was performed using the *Ac/Ds* transposable element system. A total of 378 defective kernels of various types were collected from ~1500 ears of *Ac/Ds* females. These ears were generated by crossing *Ac/Ds* females (*Ac*; *r-m3*::*Ds*; W22) to males without *Ac* activity (*r-m3*::*Ds*; W22). Viable seedlings were produced by 244 defective seeds. Obvious haploids and/or aneuploids were not observed. These plants were pollinated as females by males without *Ac* activity (*r-m3*::*Ds*; W22). Eight plants that produced >5% defective kernels as females were retested in the next generation. Reciprocal crosses were made between these eight lines and *r-m3*::*Ds*; W22 plants without *Ac* activity to determine if the defective kernel phenotype was heritable and whether it behaved as a dominant zygotic mutant or as a maternal-effect mutant. One heritable maternal-effect mutant, *hrl2*, was identified from this screen.

All mutants were tested to distinguish between maternal sporophyte and maternal gametophyte effects. Plants grown from mutant kernels were crossed as females by wild type, and the progeny of these crosses (both normal and defective) were

Table 1 Female transmission of maternal-effect mutants

Mutant	Seed types			Embryo-sac types	
	Defective kernels[a]	Normal kernel *mem*/+ heterozygotes	Normal kernel +/+ homozygotes	Mutant embryo sacs producing defective kernels (%)	Mutant embryo sacs producing no seed (%)
ssc1	32% [14–50%] (314/989)	19% (184/989)	49% (491/989)	~64	same as WT
Mn-Uq	28% [20–48%] (320/1133)	21% (44/215)	51% (110/215)	~56	same as WT
tpn1	34% [28–39%] (258/757)	9% (21/229)	57% (130/229)	~68	same as WT
bsl2	26% [13–40%] (311/1210)	21% (110/518)	53% (275/518)	~52	same as WT
nol1	30% [9–52%] (240/804)	17% (39/234)	53% (125/234)	~60	same as WT
hrl1	33% [20–48%] (339/1037)	13% (34/252)	54% (136/252)	~66	same as WT
hrl2	14% [10–18%] (183/1317)	19% (40/210)	67%* (141/210)	~20	~50
sba1	15% [6–36%] (225/1507)	36% (89/249)	49% (123/249)	~30	same as WT
mrn1	41% [32–47%] (373/904)	6% (19/299)	53% (157/299)	~82	same as WT
mrn2	29% [26–36%] (222/757)	7% (53/757)	64%* (482/757)	~50	~44
mrn3	21% [7–26%] (201/974)	6% (4/68)	73%* (50/68)	~29	~63
nbe1	12% [9–17%] (415/3607)	32% (17/52)	56% (29/52)	~22%	same as WT
stt2	28% [16–46%] (406/1426)	14% (7/52)	58% (30/52)	~62%	same as WT
stt3	35% [29–39%] (254/722)	2% (17/722)	63%* (451/722)	~56%	~41
ecr1	15% [5–27%] (126/813)	8% (3/40)	77%* (31/40)	~19%	~70
ecr2	11% [9–17%] (119/1051)	14% (4/28)	75%* (21/28)	~15%	~67

mem/+ female × +/+ male. Number of kernels in parenthesis. * $P < 0.01$, significantly >50% by χ^2. WT, wild type.
[a] Range of percentage of defective kernels from individual ears is given in brackets.

subsequently crossed as females by wild-type pollen. This backcross design ensures any phenotypes could not be the result of recessive maternal sporophyte effect mutants. Instead, defective kernels had to be maternal gametophyte effect or dominant maternal sporophyte effect mutants. The genotype of the embryo sac is irrelevant in dominant maternal sporophyte mutants, so both normal and defective kernels would be expected to segregate heterozygous mutant and homozygous wild-type plants in a 1:1 ratio. One dominant maternal sporophyte effect mutant was identified from this test from the EMS mutagenized population (data not shown). For all of the mutants described here, the defective kernels have almost exclusively inherited the mutant allele maternally and the normal kernels inherited the wild-type allele, indicating a maternal gametophyte effect on seed development (Table 1).

When crossed as females, mutants typically segregate <50% of the defective kernels. This could result either from incomplete penetrance, reduced female transmission due to failure of some of the mutant embryo sacs to be fertilized and produce a discernible seed, or a combination of both. Progeny testing of the normal kernels distinguishes these mechanisms. All of the mutants are incompletely penetrant. On a given ear, 4–32% of the seeds are mutant heterozygotes that are normal in appearance. For some mutants, this frequency is as high as that of the defective kernels (*bsl2*, *mrn3*, *nbe1*, *hrl2*, and *sba1*). In many cases, the penetrance of the mutant phenotype is variable from one cross to another (Table 1). Whether or not there is an environmental component contributing to the penetrance of the phenotype during ear development is unknown.

Female transmission was calculated by comparing the combined frequency of all heterozygous progeny to the frequency of homozygous wild-type progeny. Six of the mutants (*mrn2*, *mrn3*, *ecr1*, *ecr2*, *hrl2*, and *stt3*) produce significantly <50% mutant heterozygotes ($P < 0.01$ by χ^2). These mutations likely cause sufficient defects in a subset of the embryo sacs to prevent their fertilization or arrest development very early before a seed is visible. For *mrn3*, *ecr1*, and *ecr2*, this is the majority of the mutant embryo sacs; while for *stt3*, *mrn2*, and *hrl2* approximately half of the mutant embryo sacs do not produce a discernible seed.

The maternal-effect mutations were tested for reduced transmission through pollen as a measure for male gametophyte defects (Table 2). Mutant heterozygotes were crossed as males to wild-type females. A 50% transmission of the mutant allele in these crosses is indicative of no effect on male gametophyte function. For the *ssc1* mutation, which is linked to the easily-scored *y1* seed marker, transmission was assayed by effects on the transmission of *y1* in repulsion to *ssc1*. Likewise, *mrn2* and *stt3* were assayed by their effects on the linked *r1* gene. All three show reduced transmission of the mutant allele compared to wild type. For the rest of the mutants, seeds were grown to maturity and progeny tested. All but four of the mutants, *hrl1*, *nol1*, *Mn-Uq*, and *sba1*, showed significantly reduced transmission through the pollen ($P < 0.01$ by χ^2). The next mildest effect was seen with *bsl2* and *hrl2*. The strongest effects were seen with *stt2*, *stt3*, *nbe1*, and *mrn3*, which all had <10% transmission.

A total of 14 of the 16 mutations were mapped to chromosomal position using backcross populations and a combination of SNP and SSR markers (Figure 2; Table S1). The maternal-effect mutants are distributed over 8 of the 10 maize chromosomes. Based on the *stt2* map position and phenotype, it is possible that *stt2* is an allele of *stt1* (Phillips and Evans 2011). Other potential allelic relations include *hrl1* and *nol1*, which map very close together on chromosome 3; as well as *hrl2* and *nbe1*, which map within the same bin on chromosome 6.

Table 2 Male transmission of maternal-effect mutants

Mutant	*mem*/+ Heterozygotes
ssc1	15%* (362/2435)
Mn-Uq	39% (31/80)
tpn1	14%* (11/78)
bsl2	33%* (60/180)
nol1	45% (119/265)
hrl1	47% (83/177)
hrl2	28%* (46/162)
sba1	42% (73/172)
mrn1	22%* (34/154)
mrn2	21%* (39/185)
mrn3	7%* (6/89)
nbe1	4%* (5/114)
stt2	8%* (10/125)
stt3	1%* (8/963)
ecr1	18%* (7/39)
ecr2	14%* (6/44)

+/+ female × *mem*/+ male. * $P < 0.01$, significantly <50% by χ^2.

Phenotype of mutant kernels

The maternal-effect phenotypes can be divided into five classes: a normal endosperm with an abnormal, or aborted, embryo; a reduced endosperm with a loose pericarp (typically with some embryo abnormalities); an etched or pitted endosperm with an abnormal embryo; an empty pericarp; and a miniature, but otherwise normal, seed (Figure 3). The *ssc1* mutant is the only one that exclusively produces kernels of the aborted embryo class (Figure 3B). While most mutants have variable expressivity, *ssc1* does not. *ssc1* has an aborted embryo every time it shows any phenotype. The most common phenotype is the loose pericarp class, which has a wide range of severity. The *Mn-Uq*, *tpn1*, *bsl2*, *nol1*, *hrl1*, *sba1*, *mrn1*, *mrn2*, *mrn3*, and *nbe1* mutants all fall into this category (Figure 1, C–M; Figure 3, C–M). The *tpn1* mutant is unique in that the reduced expansion of the endosperm is limited to a collar near the crown of the endosperm, creating a protrusion on the top of the endosperm. It is possible that this is a consequence of the genetic background—*tpn1* was the only mutant in a W23 inbred background (Figure 3D). The *mrn1* mutant more commonly produces kernels of the miniature class but these are unique in having a darker endosperm than wild type (Figure 1J; Figure 3J). The next most common phenotypic class of mutants has etched, pitted kernels; this includes *nol1*, *hrl1*, *hrl2*, *sba1*, and *nbe1*. Interestingly, *nol1* and *hrl1* endosperm defects are often limited to a groove on the abgerminal side of the kernel (Figure 1G). Many mutants also show a change in the ratio of vitreous endosperm to floury endosperm, with a reduction in vitreous endosperm being most common (Figure 3, E–G, M, and Q).

The *bsl2*, *nol1*, and *Mn-Uq* mutants all have extreme variability in the amount of endosperm in the defective seeds from different crosses (Figure 1, E and F). All three mutants can produce ears in which all of the abnormal kernels are only mildly affected (Figure 1G) or more severely affected (Figure 1, E and F). For these mutants, the most severe phenotype is the empty pericarp class, which may be the strongest version of the loose pericarp phenotype. The *mrn2* and *mrn3* kernel phenotypes are less variable from one ear to another, producing kernels of the loose pericarp, reduced endosperm class almost exclusively (Figure 1, K and L; Figure 3, K and L). The most common phenotype for the *stt2* and *stt3* mutants is a miniature endosperm with a normal embryo (Figure 1, N and O; Figure 3, N and O), similar to *stt1* (Phillips and Evans 2011). These kernels are approximately half the size of their normal siblings. In some cases, *stt3* mutants will also make kernels of the pitted class. The *ecr1* and *ecr2* mutants, like *ssc1*, have an aborted embryo, but, unlike *ssc1*, they typically have a smaller endosperm than wild type. While the miniature endosperms of *stt2* and *stt3* are typically normal in shape, the ren and pit phenotypes of most mutants are often associated with irregular growth of the endosperm, not just reduced growth. This can be seen in the uneven profile of the endosperm in cross section and invaginations present in many abnormal endosperms (*e.g.*, Figure 3G).

All of the mutants have reduced viability, with *ssc1* being the most severe (Table 3). In the least severe mutants, *stt2*, *mrn3*, and *ecr2*, approximately half of the abnormal kernels are viable. The intermediate mutants, *stt3*, *Mn-Uq*, *mrn2*, *hrl2*, *nbe1*, *ecr1*, *sba1*, *mrn1*, and *nol1*, have approximately two-thirds inviable defectives; while in the more severe mutants, *hrl1*, *bsl2*, and *tpn1*, three-quarters or more of the defective seeds are inviable. In some cases, the viability varies widely between ears, particularly for *nol1*, *bsl2*, *hrl1*, and *Mn-Uq*. This is very similar to *bsl1* which produces a lethal empty pericarp phenotype in some crosses but a viable, reduced endosperm phenotype in other crosses (Gutierrez-Marcos *et al.* 2006b). Only *ssc1* causes complete loss of viability in affected seeds. Consequently, it would not have been recoverable through the screen for single defective kernels described above. All of the rest have partial viability, which presumably allowed for their recovery in the screen. It also suggests that many true maternal-effect mutants were lost to inviability of primary defective kernels.

Effects of mutations on embryo-sac morphology before fertilization

To determine if there were any effects of these mutants on embryo-sac morphology, mature embryo sacs from heterozygotes were examined using laser scanning confocal microscopy (Table 4; Figure 4). Seven different classes of phenotypes were seen in these mutants: mutants with only normal embryo sacs, miniature embryo sacs, embryo sacs with misplaced polar nuclei, embryo sacs with abnormal antipodal cells, embryo sacs with extra cells, embryo sacs with abnormal egg cells, and embryo sacs which had arrested or aborted early in development.

Five mutants, *Mn-Uq*, *tpn1*, *hrl2*, *nbe1*, and *ecr2*, have normal embryo-sac morphology with aborted embryo sacs at a wild-type background frequency (from 0 to 10% depending on plant vigor, growth conditions, and amount of transposon activity). Abnormal egg cells were seen in *ssc1*, but in only one of the three ears tested (Table 4; Figure 4, B and R). These egg

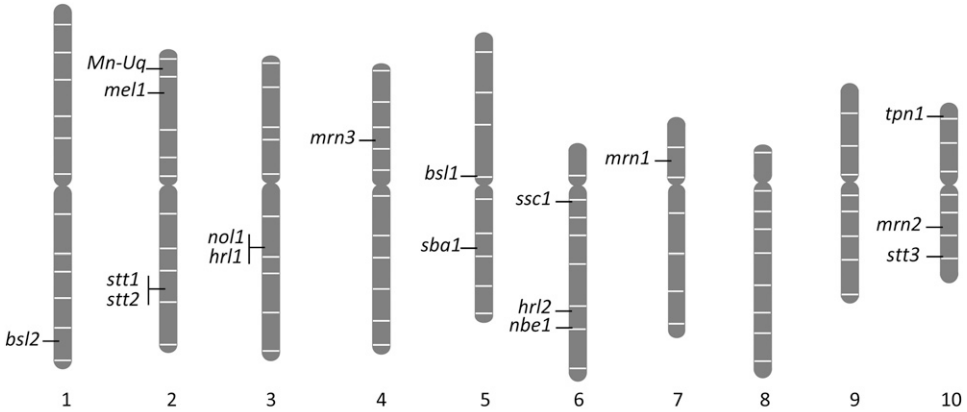

Figure 2 Map position of maternal-effect mutants. The 10 chromosomes of maize are shown. Boundaries between chromosome bins are shown with horizontal white lines. Centromeres are marked by constrictions. Approximate map positions of maternal-effect mutants (including previously published mutants) are shown. See Table S1 for supporting data.

Unmapped: *ecr1* and *ecr2*

cells have all cytoplasmic contents against the micropylar pole of the egg cell (like a synergid) rather than having the nucleus in the center of the cell surrounded by plastids and cytoplasmic strands (Figure 4R). Five mutants have misplaced polar nuclei within the central cell: *bsl2*, *nol1*, *hrl1*, *sba1*, and *mrn1* (Figure 4, C–I). In wild type, the partially fused polar nuclei are adjacent to the egg cell near the midline of the long axis of the central cell. As is the case for *bsl1* (Gutierrez-Marcos *et al.* 2006b), the polar nuclei in these mutants are almost always adjacent to each other (except for one embryo sac in *nol1*), and in *bsl2*, *nol1*, *hrl1*, and *sba1* they are adjacent to the egg cell. However, the polar nuclei are located off-center or against one of the lateral walls of the central cell. *mrn1* is exceptional in having the polar nuclei displaced toward the chalazal end of the central cell in some embryo sacs; sometimes closer to the antipodal cells than the egg cell (Figure 4H). Like *bsl1*, *sba1* typically has the polar nuclei against the future abgerminal side of the central cell (both mutants are in a W22 inbred background). *nol1*, *bsl2*, and *hrl1* (in B73, W64A, and B104 inbreds, respectively) typically have the polar nuclei against the future adgerminal side of the central cell. In addition to chalazal displacement, *mrn1* can also have the polar nuclei against the adgerminal or abgerminal side of the central cell (Figure 4I). Aborted embryo-sac development was observed in *nol1*, *hrl1*, and *bsl2*. However, based on the low frequency of this phenotype here and 50% transmission of the mutant allele through the female, this phenotype is likely unrelated to these mutations. Because *nol1* and *hrl1* map close to each other and have varying kernel phenotypes depending upon genetic background, they were examined in three different inbred lines each: B73, W23, and B104 for *nol1* and W64A, Mo17, and B104 for *hrl1*. For *nol1*, B73 had the highest frequency of misplaced polar nuclei [nearly all mutant embryo sacs (half in a heterozygote) show the phenotype] followed by W23 [about one-third of the mutant embryo sacs (one-sixth in a heterozygote) show the phenotype] and B104. In *hrl1*, polar nuclei mislocalization is seen in B104 but not in W64A or Mo17. In B104, both kernel and embryo-sac phenotypes are similar between *nol1* and *hrl1*.

Antipodal cell cluster defects were also observed in four of the mutants with polar nuclei misplacement: *hrl1*, *nol1*, *sba1*, and *mrn1* (Figure 4, E–H). *hrl1* affects the antipodal cells in the three different inbred backgrounds examined. Abnormal antipodal cell cluster morphology is shown in ~15% of the embryo sacs from heterozygotes, although the morphology varies depending upon inbred background. In W64A and B104 the antipodal cells are fewer in number but the same size as or smaller than wild type (Figure 4E), while in Mo17 the antipodal cells are also reduced in number but are larger than wild type (Figure 4F). In B104, *nol1* has an antipodal cell phenotype very similar to that of *hrl1* in W64A and B104. The antipodal cluster defects of *sba1* and *mrn1* in W22 and B73, respectively, are similar to those of *nol1* and *hrl1* in B104 (Figure 4, G–I). For *sba1* this is the more common phenotype, with polar-nuclei displacement only occurring in a subset of the mutant embryo sacs with reduced antipodal cell cluster size (one-third of the embryo sacs with antipodal cell defects also have abnormal polar nuclei position). *sba1* has no effect on polar nuclei position without an effect on antipodal cell cluster size. In *mrn1* the opposite is true; a subset of the mutants with misplaced polar nuclei also have reduced antipodal cell cluster size (one-third of the embryo sacs with abnormal polar nuclei position also have abnormal antipodal cell clusters). *mrn1* has no effect on antipodal cell cluster size when polar nuclei position is normal. These differences may result from genetic background differences or differences in the function of the mutated genes.

mrn2 and *mrn3* show a significant frequency of embryo sacs that have aborted or arrested before cellularization (Figure 4, K and L). Approximately one-third of all embryo sacs in heterozygotes (or two-thirds of the mutant embryo sacs) fail to complete development. The frequency of this phenotype is consistent with the rate of reduced female transmission in these two mutants.

stt2 and *stt3* have phenotypes similar to *stt1* (Phillips and Evans 2011). Mutant embryo sacs are smaller, with a smaller central cell and fewer antipodal cells than wild type (Figure 4, M and N). Approximately half of the *stt2*/+ embryo sacs

Figure 3 Mature kernels of maternal-effect mutants. Germinal (embryo) face (left) and a median longitudinal section (right) through the center of the embryo (or where the embryo would be). Mutant kernels are from heterozygous mutant females crossed by wild-type pollen. The embryo in the longitudinal section is oriented to the left in all kernels. (A) Wild type, (B) *ssc1*, (C) *Mn-Uq*, (D) *tpn1*, (E) *bsl2*, (F) *nol1*, (G) *hrl1*, (H) *hrl2*, (I) *sba1*, (J) *mrn1*, (K) *mrn2*, (L) *mrn3*, (M) *nbe1*, (N) *stt2*, (O) *stt3*, (P) *ecr1*, and (Q) *ecr2*. e, embryo; f, floury endosperm; v, vitreous endosperm. All kernels are shown at the same scale. Bar, 0.5 cm.

are small, suggesting that all mutant embryo sacs are affected. In contrast, only one-third of *stt3*/+ embryo sacs are small, suggesting that some *stt3* embryo sacs are normal in size. Additionally, 10% of the embryo sacs from heterozygotes *mrn2* and *stt3* mutants have extra cells or a duplication of the entire embryo sac (Figure 4, J and O), but these phenotypes were less common than the other phenotypes described above. *ecr1* mutant embryo sacs also have smaller central cells like *stt1*, *2*, and *3*; but *ecr1* is distinct from the *stt* mutants in that half of the small embryo sacs also have small, unexpanded egg cells (Figure 4, P and S).

Effects of mutations on expression pattern of a BETL marker

Because the BETL is particularly sensitive to maternal control (Charlton *et al.* 1995; Gutierrez-Marcos *et al.* 2003; Gutierrez-Marcos *et al.* 2006b), the expression of the maize BETL-specific gene reporter, $_{Pro}$*Bet1*::*GUS* (Hueros *et al.* 1999), was examined in all of the mutants to determine effects on patterning of BETL gene expression (Table 5; Figure 5; Table S2). Most crosses were made with $_{Pro}$*Bet1*::*GUS*/- hemizygotes, but some crosses were made with homozygous $_{Pro}$*Bet1*::*GUS* plants. Kernels were examined after establishment of $_{Pro}$*Bet1*::*GUS* expression near maturity [between 33 and 37 days after pollination (DAP)] for all mutants, and at 20 DAP for a few mutants [still after establishment of the normal $_{Pro}$*Bet1*::*GUS* expression pattern (compare Figure 5, A and B)]. Between 20 DAP and maturity in wild type, the $_{Pro}$*Bet1*::*GUS* expression intensifies but does not change in pattern.

The penetrance of the effects of the mutations on $_{Pro}$*Bet1*::*GUS* expression can be evaluated based on the frequency of the normal pattern of expression in the mutants (Table 5). *ssc1*, *mrn1*, *mrn2*, and *stt2* have no effects on $_{Pro}$*Bet1*::*GUS* expression; showing neither an abnormal pattern nor an

Table 3 Defective kernels that failed to produce a viable seedling

Mutant	Inviable seeds
ssc1/+	100% (108/108)
Mn-Uq/+	65% (117/180)
tpn1/+	74% (127/171)
bsl2/+	77% (132/172)
nol1/+	68% (110/162)
hrl1/+	78% (176/225)
hrl2/+	66% (83/125)
sba1/+	67% (80/120)
mrn1/+	69% (103/150)
mrn2/+	65% (66/102)
mrn3/+	43% (20/47)
nbe1/+	71% (66/93)
stt2/+	55% (33/60)
stt3/+	68% (25/37)
ecr1/+	65% (11/17)
ecr2/+	50% (7/14)

mem/+ female × +/+ male.

increased likelihood of no expression of the reporter (Table 5; Figure 5, C, K, L, and O). An abnormal pattern was seen only once out of 18 kernels expressing the transgene in *mrn3*. The next weakest effect was seen in *Mn-Uq*, *tpn1*, *stt3*, *nol1*, *bsl2*, and *hrl2*, with an effect 10–30% of the time; followed by *hrl1* and *sba1*, which affect $_{Pro}$*Bet1*::*GUS* expression more than half the time. The strongest effect was seen with *nbe1* which produced no mutant kernels with normal $_{Pro}$*Bet1*::*GUS* expression. Abnormal expression patterns can be grouped into four categories: (1) spots of expression unconnected to the base (*e.g.*, at the crown, along the back of the endosperm, or in the center of the endosperm) (Figure 5, D–F, H, I, O, and R), (2) absence of expression in a portion of the normal domain (Figure 5G), (3) expression in an expanded domain at the base of the endosperm (Figure 5, E, J, and Q), or (4) absence of expression (Figure 5N). Some mutants have expression both in extra cell layers at the base of the endosperm and in ectopic spots unconnected to this domain.

A total of 8 of the 16 mutants have expanded $_{Pro}$*Bet1*::*GUS* expression at the base of the endosperm (Table 5). This is most common in *sba1*, *hrl2*, and *ecr1*, which have this pattern in 20–28% of mutants showing this phenotype. This phenotype is also seen in *Mn-Uq*, *tpn1*, *bsl2*, and *hrl1* at a lower frequency (Table 5; and examples in Figure 5, E, F, J, and Q). *sba1* has the most extensive expansion of $_{Pro}$*Bet1*::*GUS* expression into extra layers of cells at the base of the endosperm, with expression sometimes encompassing half of the endosperm (Figure 5J). More mutants (11 out of 16) show ectopic spots of $_{Pro}$*Bet1*::*GUS* expression unconnected to any expression in the basal domain (Table 5; and examples in Figure 5, D–F, H, I, P, and R). On rare occasions, these ectopic spots are seen in the absence of any expression in the normal basal domain (once each in *stt3*, *ecr2*, and *nbe1*) (example in Figure 5R), but otherwise these spots are seen in combination with some form of basal expression. The ectopic spots have been detected most frequently in *hrl1*, *sba1*, and *stt3*, and less frequently in *nbe1*, *ecr2*, *bsl2*, *Mn-Uq*, *tpn1*, *hrl2*, *mrn3*, and *nol1*. These ectopic spots are more frequently located at the periphery of the endosperm than in the center.

Like *bsl1*, four of the mutants with misplaced polar nuclei, *nol1*, *bsl2*, *hrl1*, and *sba1* (Figure 5, F–H, and J) have abnormal

Table 4 Morphology of embryo sacs in plants heterozygous for maize maternal-effect mutations

Mutant (plants tested)	Normal	Misplaced polar nuclei	Early arrest/abortion	Small central cell	Abnormal antipodal cell cluster	Extra cells	Abnormal egg cell
ssc1/+; W22 (3)	74	0	0	0	0	0	5[a]
Mn-Uq/+; W22 (3)	61	0	0	0	0	0	0
tpn1/+; W23 (4)	51	0	4	0	0	0	0
bsl2/+; W64A (2)	36	18	1	0	0	0	0
nol1/+; B73 (4)	32	27	6	0	1	0	0
nol1/+; W23 (1)	22	4	1	0	0	0	0
nol1/+; B104 (2)	40	0	1	0	6	0	0
hrl1/+; W64A (1)	27	0	0	0	6	0	0
hrl1/+; Mo17 (1)	24	0	0	0	3	0	0
hrl1/+; B104 (2)	71	4	5	0	8	0	0
hrl2/+; W22 (1)	33	0	0	0	0	0	0
sba1/+; W22 (1)	37	8[b]	0	0	26	0	0
mrn1/+; B73 (2)	37	24[c]	0	0	9[d]	0	0
mrn2/+; W22 (1)	19	0	10	0	0	3	0
mrn3/+; W22 (2)	22	0	8	0	0	0	0
nbe1/+; W22 (3)	36	0	0	0	0	0	0
stt2/+; W22 (1)	20	0	1	20	0	0	0
stt3/+; W22 (2)	28	0	3	11	0	3[e]	0
ecr1/+; W22 (2)	45	0	2	13	0	0	5[f]
ecr2/+; W22 (2)	44	0	0	0	0	0	0

[a] Abnormal egg cells were only seen in one of three ears tested.
[b] These individuals are a subset of the embryo sacs with abnormal antipodal cells.
[c] The chalazal displacement of polar nuclei was seen in one of two ears tested, but the lateral displacement of polar nuclei was seen in both ears.
[d] These individuals are a subset of the embryo sacs with misplaced polar nuclei.
[e] Extra cells were only seen in one of two ears tested.
[f] These individuals are a subset of the embryo sacs with small central cells.

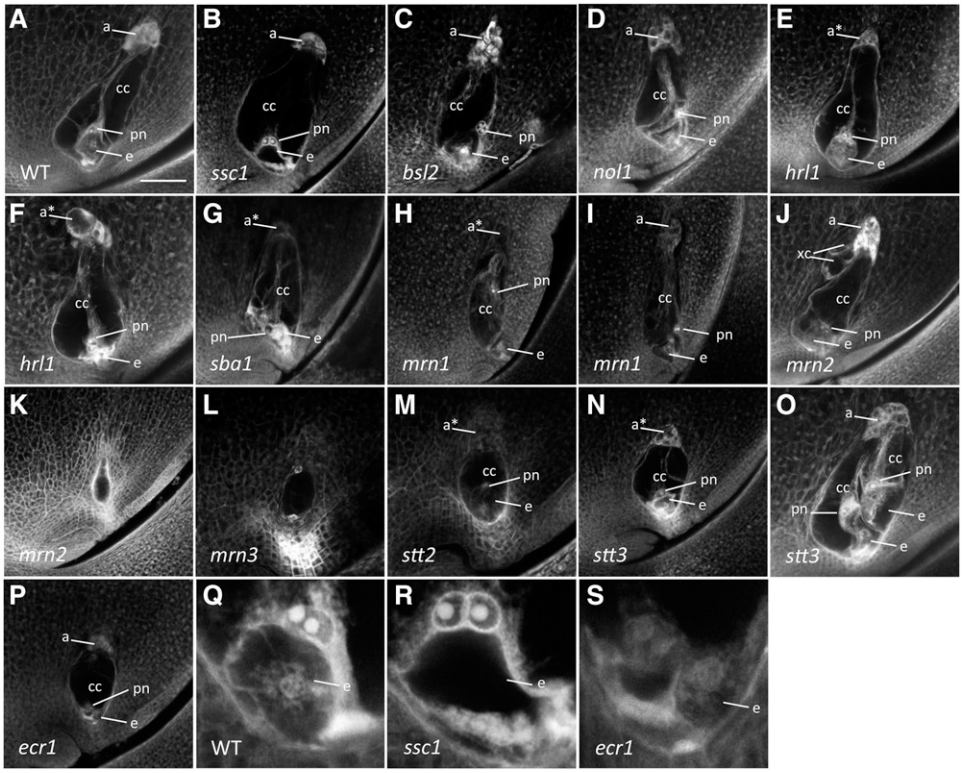

Figure 4 Mature embryo-sac phenotypes in plants heterozygous of maternal-effect mutants. The micropylar end is at the bottom and the chalazal end at the top of each panel. The future germinal side of the kernel is oriented toward the right of each panel. (A and Q) Wild type. (B) *ssc1*. (C) *bsl2* with polar nuclei against the future germinal side. (D) *nol1* with polar nuclei against the future germinal side. (E) *hrl1* in W64A with few normal sized antipodal cells. (F) *hrl1* in Mo17 with few, large antipodal cells. (G) *sba1* with polar nuclei against the future abgerminal side and few antipodal cells. (H) *mrn1* with polar nuclei near the chalazal end of the central cell and with few antipodal cells. (I) *mrn1* with polar nuclei against the future germinal side. (J) *mrn2* with extra cells near the antipodal cell cluster. (K) *mrn2* embryo sac arrested at 2-nucleate stage. (L) *mrn3* embryo sac arrested at the 2-nucleate stage. (M) *stt2* small embryo sac with few antipodal cells like *stt1*. (N) *stt3* small embryo sac with few antipodal cells like *stt1*. (O) *stt3* with duplicated embryo sac. (P) *ecr1* small embryo sac with small egg cell. (Q) Close up of wild-type egg cell. Note enlarged egg cell with centrally positioned nucleus surrounded by cytoplasmic strands. The brightly fluorescent material next to the egg cell is the remnant of a synergid. (R) Close up of egg found in *ssc1*. The egg cell has expanded, but all cytoplasmic contents are at the micropylar end like an immature synergid. (S) Close up of small egg cell in *ecr1*. (A–P) Bar, 100 μm. (Q–S) Bar, 20 μm. a, antipodal cell cluster; a*, abnormal antipodal cell cluster; cc, central cell; e, egg cell; pn, polar nuclei; WT, wild type; xc, extra cells.

patterns of expression of the $_{Pro}$*Bet1*::*GUS* reporter. *nol1* is more likely to lack expression within the basal region of the endosperm, while *bsl2*, *hrl1*, and *sba1* are more likely to have ectopic expression outside of the basal domain. Kernels that lack expression in the central domain but have it in the abgerminal and adgerminal domains—a pattern that is common in *bsl1*—are rare in these mutants. These differences may be a consequence of the polar nuclei typically being placed on the future abgerminal side of the central cell in *bsl1*, but on the future adgerminal side of the central cell in *nol1*, *hrl1*, and *bsl2*. *hrl1* and *sba1* did not produce kernels with partial $_{Pro}$*Bet1*::*GUS* expression patterns, unlike *bsl1*, *bsl2*, and *nol1*. All four of these mutants show expression of $_{Pro}$*Bet1*::*GUS* outside of the normal BETL domain, with *sba1* having the strongest effect. *mrn1* is unique in having an effect on polar nuclei position but no effect on $_{Pro}$*Bet1*::*GUS* expression (Table 5; Figure 5K). Whether all of the differences between these mutants are a consequence of differences in genetic background or differences in the functions of the mutated genes is unclear.

In *nbe1* mutants, expression of $_{Pro}$*Bet1*::*GUS* was never detected in the normal basal domain in the 32 mutant kernels examined, and only once in ectopic puncta outside of this domain (Table 5; Figure 5N). The *hrl1* and *sba1* mutants also prevent $_{Pro}$*Bet1*::*GUS* expression in some kernels (*i.e.*, defective kernels lack expression more frequently than their wild-type siblings) (Table 5).

Discussion

Both mutant analyses (Gavazzi *et al.* 1997; Evans and Kermicle 2001; Grini *et al.* 2002; Olsen 2004; Köhler and Grossniklaus 2005; Pagnussat *et al.* 2005; Gutierrez-Marcos *et al.* 2006b; Pien and Grossniklaus 2007; Phillips and Evans 2011; Del Toro-De León *et al.* 2014) and gene expression studies (Vielle-Calzada *et al.* 2000; Baroux *et al.* 2001; Grimanelli *et al.* 2005; Autran *et al.* 2011; Baroux and Grossniklaus 2015) indicate a significant amount of both endosperm and embryo development is under maternal control. Differential timing of paternal allele activation would allow for some processes of embryogenesis to be controlled by the maternal genome longer than others.

In this study, we describe 16 maternal-effect mutations. Most of these cause morphological defects in the embryo sac prior to fertilization. Consequently, these mutations are likely affecting production of gametes with the correct structure to support normal development of the seed rather than affecting individual imprinted genes, or the imprinting process itself. Subcellular organization of the central cell in particular appears important for endosperm development. A connection between antipodal cell development and seed development is implied by some mutant phenotypes as well. These mutations identify at least 11 loci not previously indicated to play a role in the maternal regulation of seed development. Based on map

Table 5 Endosperm $_{Pro}$*Bet1*::*GUS* expression in maternal-effect mutants

Normal $_{Pro}$*BET1*::*GUS* expression in defective kernels[a]	Frequency of kernels expected to have $_{Pro}$BET1::GUS but lacking it in the BETL	Frequency of kernels with $_{Pro}$BET1::GUS only in part of the BETL	Frequency of kernels with $_{Pro}$BET1::GUS in extra cell layers	Frequency of kernels with $_{Pro}$BET1::GUS in ectopic spots
100% normal	*nbe1* (100%),	*nol1* (22%),	20–28%	10–25%
ssc1 (47)	*hrl1* (38%),	*bsl2* (6%)	*ecr1*,	*stt3*,
mrn1 (47)	*sba1* (36%)		*hrl2*,	*hrl1*,
mrn2 (77)			*sba1*	*sba1*
stt2 (35)				*bsl2*,
94–96% normal				*Mn-Uq*,
mrn3 (42)				*tpn1*,
60–85% normal				*ecr2*
ecr2 (26)				
tpn1 (41)				
Mn-Uq (30)				
stt3 (30)				
nol1 (67)				
ecr1 (33)				
bsl2 (59)				
hrl2 (39)				
25–45% normal			<10%	<10%
hrl1 (60)			*Mn-Uq*,	*mrn3*,
sba1 (63)			*tpn1*,	*hrl2*,
0% normal			*bsl2*,	*nol1*,
nbe1 (32)			*hrl1*	*nbe1*

See Table S2 for supporting data.
[a] Total defective kernels tested for expression in parentheses.

position and phenotype, *stt2* could be an allele of *stt1*, and *nol1* and *hrl1* could be allelic with each other. *hrl2* and *nbe1* map within the same bin on chromosome 6 but have distinct seed and transmission phenotypes within the same inbred background, and so are less likely than the other two pairs to be allelic with each other. Because the action of these mutations is during the haploid phase of the life cycle, complementation tests are very difficult. Fine mapping and cloning of these genes will determine if they are indeed allelic. Although all of the mutants behave as maternal effects rather than zygotic dominants, *mrn2* and *mrn3* have dominant effects on sporophyte development. Tassels of *+/mrn2* mutants have expanded glumes, giving them a brush-like appearance, and the tassels and ears of *+/mrn3* mutants are smaller than wild type (Figure 1L; Figure S3). The mutant phenotypes in the sporophyte could either be caused by haploinsufficiency or gain-of-function mutations, raising the possibility that *mrn2* and *mrn3* may be causing gametophyte defects by gain of function rather than loss of function of the affected genes.

These maternal-effect mutants include several different types of effects on seed development and several classes of prefertilization embryo-sac defects. Although mutants were selected solely for their maternal effects, the majority also affect the male gametophyte as measured by reduced transmission of the mutant allele. The nature of the defects in the pollen grain are unknown, but for some mutants the effect is very severe with <10% male transmission efficiency. These results are in agreement with other screens for gametophyte mutants that indicate most mutants affect both sexes (summarized in Evans and Grossniklaus 2009).

Maternal-effect mutants usually affect both the endosperm and the embryo, but there are examples of specific effects on one or the other. Mild effects on the endosperm, such as a miniature kernel, often have no effect on the embryo. Severe endosperm defects, however, are almost always associated with abnormal embryo development or reduced viability. Whether the effects on the embryo for most of the mutants indicate a direct role for the mutated gene in embryo development or a downstream consequence of the embryo growing in the context of an abnormal endosperm is unclear. Severe defects in embryo development, in contrast, can occur in the absence of visible defects in the endosperm; although subtle defects in the ability of the endosperm to support growth of the embryo in these cases cannot be ruled out.

Many maternal-effect mutants are also associated with embryo-sac abnormalities prior to fertilization. Only a third of them have no morphological defects prior to fertilization. These defects fall into six distinct classes: misplacement of the polar nuclei within the central cell, abnormal antipodal cell cluster morphology or size, abnormal egg cell size/morphology, reduced embryo-sac size (particularly of the central cell), embryo sacs with extra cells, and embryo-sac arrest/abortion. Mutants can express multiple phenotypes, and phenotypes can vary depending upon genetic background. Some phenotypes have only been seen in one individual per mutant, and, for these, there is less confidence that they are caused by the mutation being analyzed. Examples of this are the abnormal egg cells in *ssc1*, the extra cells in *stt3*, or the chalazal displacement of polar nuclei in *mrn1*. These effects may be caused by a

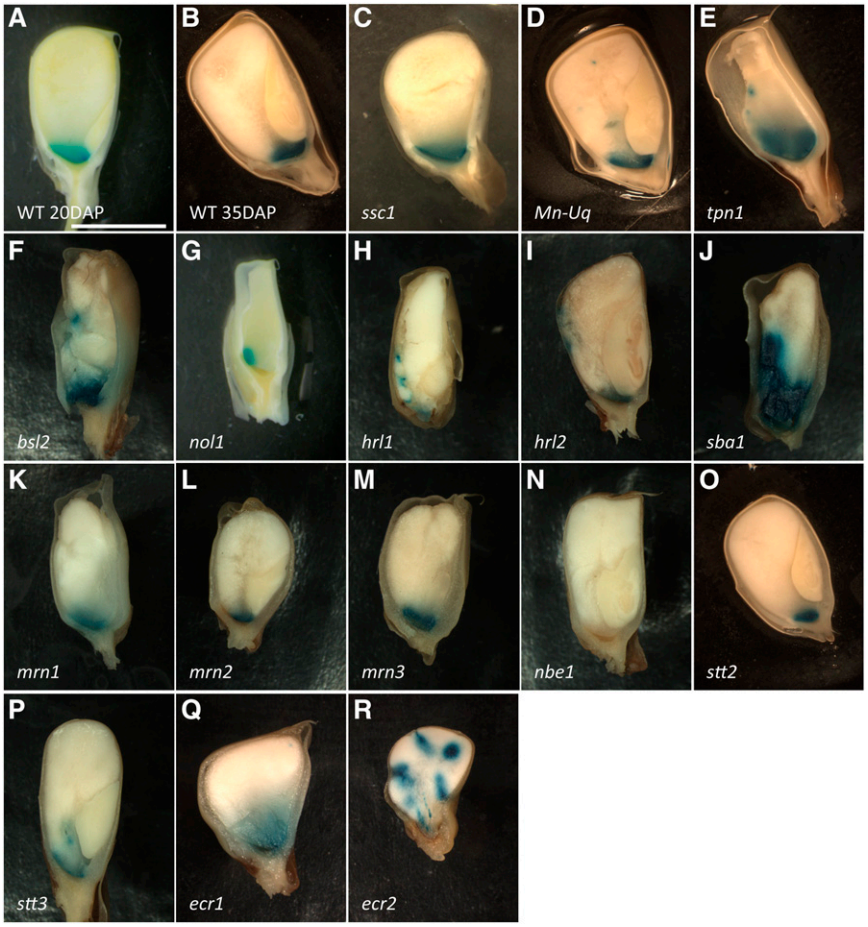

Figure 5 Pattern of expression of *pBET1*::*GUS* in maternal-effect mutants. (A) Wild type 20 DAP, (B) wild type 35 DAP, (C) *ssc1*, (D) *Mn-Uq*, (E) *tpn1*, (F) *bsl2*, (G) *nol1*, (H) *hrl1*, (I) *hrl2*, (J) *sba1*, (K) *mrn1*, (L) *mrn2*, (M) *mrn3*, (N) *nbe1*, (O) *stt2*, (P) *stt3*, (Q) *ecr1*, and (R) *ecr2*. (B–F and H–R) Kernels are between 33 and 37 DAP. (A and G) Kernels are 20 DAP. Kernels are shown at the same scale. Bar, 0.5 cm. WT, wild type.

second mutation segregating in each line, or they may depend on a particular growth condition.

Misplacement of polar nuclei and reduced central cell size have previously been shown to occur in maize maternal-effect mutants (Gutierrez-Marcos *et al.* 2006b; Phillips and Evans 2011). Three additional mutants with small central cells and small seeds were identified here: *stt2*, *stt3*, and *ecr1*. This aspect of the phenotype is very similar to that of *stt1*. Indeed, *stt2* may be an allele of *stt1*, because these mutants map within the same chromosomal region. *stt3* and *ecr1* have additional kernel phenotypes not seen in *stt1* or *stt2*. *stt3* shows occasional ectopic expression of *pBet1*::*GUS* expression, and *ecr1* kernels are typically germless. Both of these mutants also have embryo-sac phenotypes not seen in *stt1* or *stt2*. In one of the two individuals tested, *stt3* had embryo sacs with extra cells or ovules with a complete embryo-sac duplication. Half of the small *ecr1* embryo sacs also have a small egg cell. The abnormal eggs of *ecr1* might be unable to produce normal embryos in the seed, and may suggest that even the eggs without visible abnormalities are affected, since the frequency of germless kernels is higher than the frequency of egg defects. Since egg abnormalities segregated in one *ssc1* ear, defects in the egg cell may contribute to embryo arrest in this mutant as well.

Five mutants have misplaced polar nuclei like *bsl1* (Gutierrez-Marcos *et al.* 2006b). Four of these *bsl2*, *nol1*, *hrl1*, and *sba1* cause abnormal patterning of $_{Pro}$*Bet1*::*GUS* expression, also like *bsl1*. These results support the model that there is a prepattern of an unknown factor(s) within the central cell that controls BETL development and that it is disrupted in these mutants. Alternatively, these genes may have multiple, independent roles during embryo-sac and seed development. For all four of these mutants, the effects on $_{Pro}$*Bet1*::*GUS* can be more severe than in *bsl1*. The exception to this effect is *mrn1*. *mrn1* has no effect on $_{Pro}$*Bet1*::*GUS*, despite affecting polar nuclei placement. Therefore, the abnormal $_{Pro}$*Bet1*::*GUS* pattern is not a direct consequence of the abnormal position of the polar nuclei themselves. Several mutations have normal embryo-sac morphology but still affect $_{Pro}$*Bet1*::*GUS* expression pattern: *Mn-Uq*, *tpn1*, *nbe1*, *ecr2*, and *hrl2*. These mutations could be in genes required either before or after fertilization for establishing or maintaining such a BETL-determining pattern. Consequently, polar nuclei movement and the placement of this hypothetical determinant are genetically separable, with some regulatory factors shared between the two processes but others unique to one or the other.

Two types of misexpression of $_{Pro}$*Bet1*::*GUS* were observed: expression in extra cell layers of the endosperm, and in ectopic spots in the endosperm. Expression in extra cell layers at the base of the endosperm could be interpreted as a conversion of the domains adjacent to the BETL, the CZ,

and/or the BIZ, into BETL cells. More experiments are required to determine the extent to which markers for all three domains (Li *et al.* 2014) are affected in these mutants. The ectopic spots of $_{Pro}$*Bet1*::*GUS* are more commonly found at the periphery of the endosperm than the interior, possibly indicating a bias toward BETL formation at a boundary. Ectopic spots of expression are also much more common on the abgerminal side of the endosperm than the germinal side, suggesting there is inhibition of BETL gene expression near the embryo. Bias for BETL formation away from the center and toward the abgerminal side of the endosperm is consistent with the effects of overexpressing *meg1* (Costa *et al.* 2012).

All of the mutants with misplaced polar nuclei, except for *bsl2*, also have abnormal antipodal cell clusters. The most common phenotype is a reduced number of antipodal cells of normal morphology. In *hrl1* and *nol1*, effects on the antipodal cells are background dependent. In *hrl1*, the antipodal cells are fewer in number but normal in appearance in W64A and B014, but larger than wild type in Mo17. In *nol1*, the antipodal cells are usually normal in W23 and B73 but more frequently have reduced numbers in B104. *nol1* and *hrl1* have similar kernel and embryo-sac phenotypes in B104, which, coupled with their map positions, is consistent with them having mutations in the same gene. They also have similar transmission frequencies and are both likely homozygous viable (data not shown).

The antipodal cells have been proposed to transfer nutrients to the embryo sac (or early seed) or to act as a signaling center marking one pole of the endosperm (Diboll 1968; Chettoor and Evans 2015). The maize *rgh*-1210* mutant indicates a connection between endosperm/embryo development and postfertilization morphology of the antipodal cells, although the cause-and-effect relationship of these phenotypes is undetermined (Clark and Sheridan 1988). In the maternal-effect mutants described here, the frequency of the antipodal cell phenotypes is often lower than that of the seed phenotypes. Consequently, there is not a 1:1 correspondence between seed phenotype and antipodal cell cluster phenotype. Reduced antipodal cell cluster size may contribute to reduced seed growth or abnormal endosperm patterning after fertilization, but the correlation is not clear. It is equally possible that the mutated genes promote proliferation of all cells, including the antipodals and the endosperm after fertilization. In this model, the maternal effect could be caused by delayed activation of the paternal allele. Under this model the mutations affect antipodal cell and seed development independently through the same mechanism, rather than affecting seed development via effects on antipodal cell development.

While a subset of these mutations may affect imprinting or be in imprinted genes, the majority seem to be involved in producing gametes with the correct structure to support normal development of the endosperm and/or embryo. While it cannot be ruled out that the mutants with abnormal gamete morphology also affect imprinting, the simplest model is one in which abnormal seed development is a direct consequence of gamete abnormalities. More data will be necessary to distinguish between these possibilities. Mutants with normal embryo-sac morphology (*Mn-Uq*, *tpn1*, *hrl2*, *nbe1*, and *ecr2*) are better candidates for affecting imprinted genes. These effects could be caused by mutations in genes with delayed or no paternal allele activation in the endosperm (or embryo) or with global effects on imprinting. These alternatives can be resolved once the mutant genes have been identified.

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Note added in proof: See Bai *et al.* 2016 (pp. 221–231) in this issue, for a related work.

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Maternal Gametophyte Effects on Seed Development in Maize

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Figure S1. Example of a single defective kernel from a *Mu* active *UniformMu* W22 female (arrowhead).

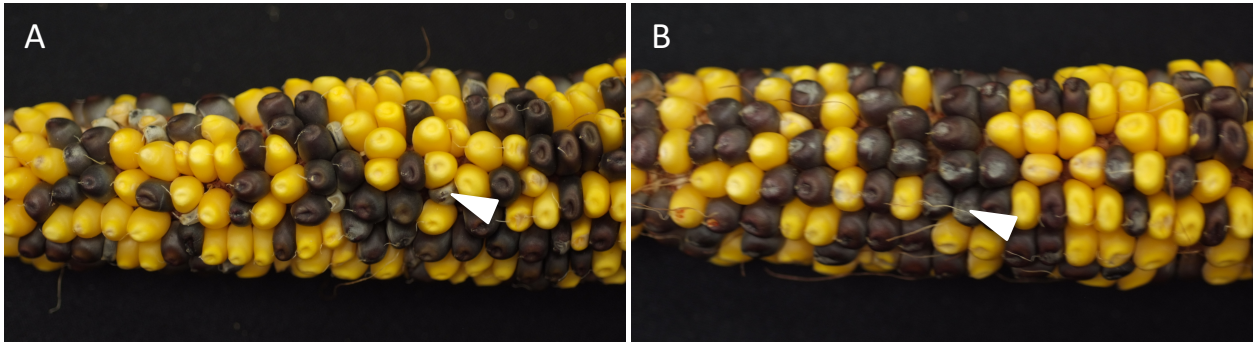

Figure S2. Example of a dominant mutant isolated from the maternal effect screen. (A) Mutant *+/dek*-N6* female by wild type pollen. (B) Wild type female by *+/dek*-N6* pollen. Note defective kernels segregating in both crosses, although the phenotype is more severe as a female than as a male indicating a potential dosage effect. Arrowheads indicate representative defective kernels on each ear.

Figure S3. Tassel phenotypes of *mrn2* and *mrn3*. (A) Tassel of wild-type W22. (B) Tassel of *+/mrn2* W22 heterozygote. (C) Tassel of *+/mrn3* W22 heterozygote.

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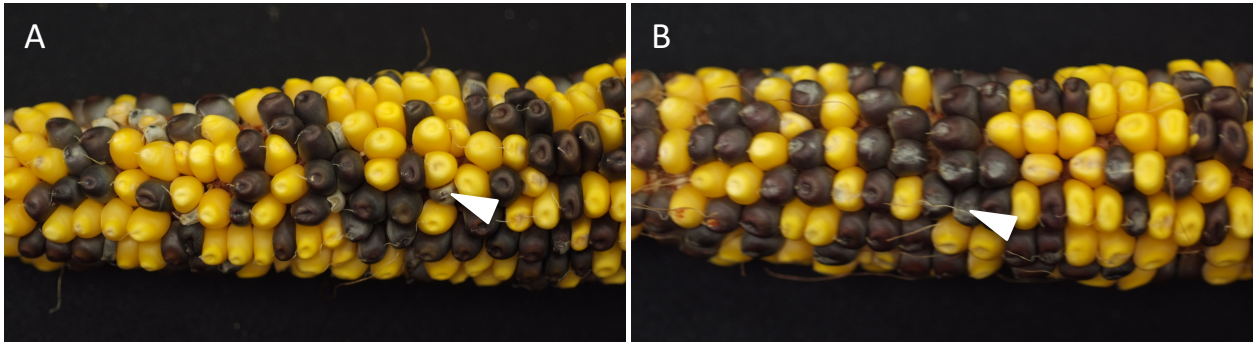

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Table S1 Mapping data for maternal effect data. (.xlsx, 48 KB)

Available for download as a .xlsx file at
www.g3journal.org/lookup/suppl/doi:10.1534/genetics.116.191833/-/DC1/TableS1.xlsx

Table S2 proBET1::GUS expression data. (.xlsx, 36 KB)

Available for download as a .xlsx file at
www.g3journal.org/lookup/suppl/doi:10.1534/genetics.116.191833/-/DC1/TableS2.xlsx